Progress Report

DE-FG02-07ER64490

Engineering Coordination of Regulatory Networks and Intracellular Complexes to Maximize Hydrogen Production by Phototrophic Microorganisms

James C. Liao, UCLA

This project is a collaboration with F. R. Tabita of Ohio State. Our major goal is to understand the factors and regulatory mechanisms that influence hydrogen production. The organisms to be utilized in this study, phototrophic microorganisms, in particular nonsulfur purple (NSP) bacteria, catalyze many significant processes including the assimilation of carbon dioxide into organic carbon, nitrogen fixation, sulfur oxidation, aromatic acid degradation, and hydrogen oxidation/evolution. Our part of the project was to develop a modeling technique to investigate the metabolic network in connection to hydrogen production and regulation.

Organisms must balance the pathways that generate and consume reducing power in order to maintain redox homeostasis to achieve growth. Maintaining this homeostasis in the nonsulfur purple photosynthetic bacteria is a complex feat with many avenues that can lead to balance, as these organisms possess versatile metabolic capabilities including anoxygenic photosynthesis, aerobic or anaerobic respiration, and fermentation. Growth is achieved by using H_2 as an electron donor and CO_2 as a carbon source during photoautotrophic and chemoautotrophic growth, where CO_2 is fixed via the Calvin-Benson-Bassham (CBB) cycle. Photoheterotrophic growth can also occur when alternative organic carbon compounds are utilized as both the carbon source and electron donor. Regardless of the growth mode, excess reducing equivalents generated as a result of oxidative processes, must be transferred to terminal electron acceptors, thus insuring that redox homeostasis is maintained in the cell. Possible terminal acceptors include O_2, CO_2, organic carbon, or various oxyanions. Cells possess regulatory mechanisms to balance the activity of the pathways which supply energy, such as photosynthesis, and those that consume energy, such as CO_2 assimilation or N_2 fixation. The major route for CO_2 assimilation is the CBB reductive pentose phosphate pathway, whose key enzyme is ribulose 1,5-biphosphate carboxylase/oxygenase (RubisCO). In addition to providing virtually all cellular carbon during autotrophic metabolism, RubisCO-mediated CO_2 assimilation is also very important for nonsulfur purple photosynthetic bacteria under photoheterotrophic growth conditions since CO_2 becomes the major electron sink under these conditions.

In this work, Ensemble Modeling (EM) was developed to examine the behavior of CBB-compromised RubisCO knockout mutant strains of the nonsulfur purple photosynthetic bacterium *Rhodobacter sphaeroides*. Mathematical models of metabolism can be a great aid in studying the effects of large perturbations to the system, such as the inactivation of RubisCO. Due to the complex and highly-interconnected nature of these networks, it is not a trivial process to understand what the effect of perturbations to the metabolic network will be, or vice versa, what enzymatic perturbations are necessary to yield a desired effect. Flux distribution is controlled by multiple enzymes in the network, often indirectly linked to the pathways of interest. Further, depending on the state of the cell and the environmental conditions, the effect of a perturbation may center around how it effects the carbon flow in the network, the balancing

of cofactors, or both. Thus, it is desirable to develop mathematical models to describe, understand, and predict network behavior. Through the development of such models, one may gain the ability to generate a set of testable hypotheses for system behavior.

Early advancement in the field of kinetic metabolic modeling was accomplished through the development of Metabolic Control Analysis (MCA). MCA has been used successfully in many studies to identify portions of the network that exhibit control over the metabolic flux, and a thorough explanation of the facets of MCA has been described. The practical use of MCA requires the implementation of many dedicated experiments unrelated to the normal course of strain design to determine the control coefficients. The theoretical derivation of MCA comes from a local linearization of the system around the reference steady state, and thus the equations for the control coefficients and the related parameters are an outcome of infinitesimal perturbations to the system and may or may not be indicative of the result of large perturbations to highly non-linear biological systems, such as the inactivation of enzymes or implementation of new pathways in the network. For situations where many of the parameters are unknown, an effective Monte Carlo algorithm presented in the framework of MCA under uncertainty has also been introduced. In this work, a strategy of sampling enzyme elasticities is employed to account for uncertainty in kinetic parameters, and thus a distribution of the possible control coefficients can be obtained, even in the absence of well-characterized kinetics. Alternative developments to MCA have been carried out that allow for experimental data to be used to deduce additional parameters related to large changes to the metabolic network, including the introduction of variable elasticities. Other avenues of developing kinetic models have included the area of deducing kinetic parameters and constructing deterministic ordinary differential equation (ODE) models using approximations of the enzyme kinetics that can capture the dynamic behavior of the system, such as by using irreversible Michaelis-Menten expressions. However, many of the parameters needed are unknown and require time-dependent metabolite concentration data from experiment in order to optimize the parameters that best capture the dynamic concentration changes in metabolite levels. Stochiometric and thermodynamic constraints using mass-action kinetics have been used to construct the possible space of kinetic parameters (15), but then finding the parameters that fit one's system still requires an experimental characterization of the system.

However, in EM, the hurdle of quantifying detailed enzyme kinetics of each reaction in the system is avoided, and instead an ensemble of models that all reach the given steady state is constructed. These models span the space of kinetics allowable by thermodynamic constraints, and are based on elementary reactions, which are the most fundamental and general kinetic descriptions for enzymatic reactions. Elementary reactions are advantageous, as they can be transformed into a set of log-linear equations, which requires mass-action kinetics, and is not valid if enzyme concentrations are not explicitly considered. However, by forming the elementary reactions as mass-action kinetics at the enzyme, the log-linear transformation can be completed while still preserving the intrinsic non-linear behavior in enzyme kinetics, thus preserving the true biological mechanism. This framework does not rely on a local linearization of the system, and one is free to perform and determine the effect of large perturbations on the network. Through this approach, the problem of acquiring detailed kinetic parameters is circumvented, yet the generated models capture phenotypes that are dependent on kinetics.

In this work, we implemented the EM approach to the metabolism of *R. sphaeroides.* In the presence of a non-functional CBB pathway, *R. sphaeroides* has evolved two alternative routes through spontaneous adaptive mutations to dissipate the excess reducing power and maintain redox homeostasis and allow photoheterotrophic growth. Redox homeostasis can be achieved either by activation of the pathways for the expression of the nitrogenase complex, even in the presence of ammonia, thus dissipating excess reducing power via the hydrogenase activity of this enzyme to produce large quantities of hydrogen gas (25, 38), a biofuel of large significance (9). Alternatively, we have found that *R. sphaeroides* may express a sulfate reduction pathway that also dissipates excess reducing power via the formation of hydrogen sulfide. EM correctly showed that upon the inactivation of RubisCO, causing the CBB pathway to be nonfunctional, no models within the ensemble were able to maintain redox homeostasis. However, when either of the above mentioned pathways were activated, a subset of models within the ensemble restored redox homeostasis in the cell, while also correctly predicting a significant drop in the growth rate and uptake rate of the carbon source malate. Here we demonstrate that *R. sphaeroides* is capable of evolving alternative ways to dissipate excess reducing power under conditions where the CBB pathway is made inactive, and that Ensemble Modeling is able to capture and describe this behavior.

Publications:

1) Tran, L.M.; Rizk, M.L.; and J.C. Liao (2008) Ensemble Modeling of Metabolic Networks. Biophys J. 95(12):5606-17

2) Contador, C.A.; Rizk, M.L.; Asenjo, J.A.; and Liao, J.C. (2009) Ensemble modeling for strain development of L-lysine-producing Escherichia coli. Metab. Eng. [Epub ahead of print] PMID: 19379820

3) Rizk ML, Laguna R, Smith KM, Tabita FR, Liao JC. (2011) Redox homeostasis phenotypes in RubisCO-deficient Rhodobacter sphaeroides via ensemble modeling. *Biotechnol Prog*. 27:15-22